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Review

Strategies for the identification of non-polar toxicants in aqueous environmental samples using toxicity-based fractionation and gas chromatography–mass spectrometry

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ABSTRACT

Toxicity-based fractionation is a useful tool for the isolation and identification of non-polar organic compounds that are present at toxic concentrations in aqueous environmental samples. Methods for isolating such toxicants from the aqueous sample matrix and techniques for fractionating the compounds for the purpose of reducing the complexity of the sample matrix and thus facilitating identification are evaluated. Strategies for analyzing gas chromatographic–mass spectrometric data and confirming toxicant identification are presented. Studies that use toxicity-based fractionation for identifying the cause of toxicity in aqueous environmental samples such as municipal and industrial wastewater treatment plant effluents and ambient waters are discussed.

CONTENTS

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LIST OF ABBREVIATIONS

C_{18}	Octadecyl
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IR	Infrared
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
SPE	Solid-phase extraction
UV	Ultraviolet

1. INTRODUCTION

A major goal in the environmental sciences is the identification of compounds that are present in the environment at toxic concentrations. This has proved to be a difficult task, however, because environmental samples have been found to be chemically complex, containing hundreds or even thousands of chemical compounds. This complexity makes it very difficult to "find and identify" the toxicants. Of all these compounds, only a very few may cause the observed toxicity in an environmental sample. One approach that has been developed for reducing the complexity of environmental samples is toxicity-based fractionation. The goal in this approach is to separate those compounds that are causing toxicity from those that are not causing toxicity before carrying out chemical analysis. In the toxicity-based approach a toxic environmental sample is chemically fractionated into several subsamples (fractions). The toxicity of each fraction is determined. In this way, the fraction that contains the compounds causing toxicity is identified. Subsequently, successive chemical fractionations coupled with toxicity tests can be performed, ideally resulting in the isolation of the compounds causing toxicity in a fraction that is chemically very much less complex than the original environmental sample. Such a simplified matrix can greatly facilitate toxicant identification by analytical techniques such as gas chromatography–mass spectrometry (GC–MS) [1].

This approach has been used to identify many kinds of environmental and health hazards in many different kinds of environmental samples. It originated with testing for mutagenic compounds in complex samples such as cigarette smoke [2]. It has been applied to samples such as air particulates [3–5], effluents [6], and sediments [7,8] to isolate mutagens using bacteria as the test species. Also, naturally occurring insect repellents have been isolated from plants by using leafcutter ants [9]. Shellfish poisons have been isolated with mice as the test species [10]. The toxicity-based approach has also found utility [1,11–14] in the identification of toxicants in environmental aqueous samples such as municipal and industrial wastewater treatment plant effluents and in ambient water using aquatic spe-

cies such as *Ceriodaphnia dubia, Daphnia magna* and *Pimephales promelas.*

While the toxicity-based approach has been used to isolate many different kinds of biologically active compounds in many different types of samples the scope of this review is limited to the isolation and identification of compounds present in concentrations that are toxic to aquatic organisms in aqueous environmental samples such as municipal and industrial wastewater treatment plant effluents and ambient waters. Selected research [1,11–24] will be discussed in this review that deals with compounds that are at acutely toxic concentrations in samples of this kind. Potential toxicants in such samples can include cationic metals, anionic inorganic compounds, chlorine, ammonia and polar and non-polar organic compounds. The scope of this review, however, will include only the identification of non-polar organic compounds that are present in sufficiently high concentrations to exert toxic effects on aquatic organisms. In this discussion, non-polar organic compounds are considered to be those whose octanol/water partition coefficient (log K_{ow}) is approximately 2 or greater. Strategies for the isolation of toxicants from an aqueous matrix, the fractionation of these isolated compounds, and the identification of these compounds using GC–MS will be addressed. A comparison of a selected number of these strategies is presented in Table 1.

2. TOXICITY-BASED FRACTIONATION

In a toxicity-based fractionation the test organism used in the toxicity test is the "detector" of the compounds that are causing toxicity (toxicants) in a sample or sample fraction. Until the toxicants are identified this is the only way to detect them. Because the response of the test organism is used to direct the fractionation, certain limitations are imposed on the chemical methods and materials that can be used to carry out the fractionation. Solvents and sorbents used for chemical separations can impart artifactual toxicity to samples and sample fractions. Choice of isolation and fractionation techniques should be made with this concern in mind. When artifactual toxicity is added, "tracking" of toxicity is negated. To track toxicity is to use the test organism to detect the toxicant(s) as it progresses through successive fractions in a fractionation scheme until it is sufficiently isolated to be identified by analytical means. Fig. 1 illustrates a toxicity-based fractionation scheme used by Burkhard *et al.* [1] to isolate and identify toxicants in a municipal wastewater treatment plant effluent. Here it can be seen that toxicity tests are used to detect the presence of the toxicant(s) in the sample and in sample fractions. The toxicity of an aqueous environmental sample can be measured directly with an aquatic organism while the toxicity of a sample fraction usually cannot, because the matrix of the sample is aqueous while the matrix of a fraction is very often an organic solvent. In the fractionation of non-polar organic compounds, the compounds are extracted from the aqueous sample and are separated into subsamples (fractions) with an organic solvent matrix. This has important implications on the ability to detect sample toxicants in the fractions, because most undiluted organic solvents are acutely toxic to aquatic organisms and would therefore add artifactual toxicity to the extract or fraction. When artifactual toxicity is added to a fraction or extract the test organism cannot differentiate between sample toxicity and artifactual toxicity. Therefore the extract or fraction must be diluted with water until the solvent is not longer at toxic concentrations or the solvent must be removed from the extract or fraction before toxicity testing can take place. The merits and drawbacks of both approaches will be discussed in detail in later sections.

To ensure that artifactual toxicity does not confound toxicant identification, procedural blanks must always be run alongside the environmental sample. The primary purpose of this blank is to indicate whether any toxicity is being imparted by the fractionation procedure itself, *i.e.* from solvents or sorbents. The blank should be run through all the steps from isolation of non-polar organic compounds, through the fractionation procedures, right through analysis by

TABLE I

SELECTED TOXICITY-BASED FRACTIONATION SCHEMES USED FOR ISOLATING AND IDENTIFYING TOXICANTS IN AQUEOUS ENVIRONMENTAL SAMPLES

Ref.	Sample matrix	Toxicity test/ organism	Method of isolating non-polar organic toxicants from aqueous sample matrix	Method of fractionating non-polar toxicants	Multi-level fractionation	Toxicant identification	
						Tentative	Definitive
17	Industrial effluent	48 h EC50 *D. pulex*	Liquid–liquid solvent extraction	Liquid–liquid acid–base fractionation	None	Yes	No
23	Petroleum refinery effluent	96 h LT50 *D. magna*	Liquid–liquid solvent extraction	Liquid–liquid acid–base fractionation	Silica gel chromatography	Yes	No
16	Surface river water	24 h EC50 *D. magna*	XAD-2, XAD-7 SPE	None	None	Yes	No
14	Industrial effluent	48 h LC50 *C. dubia;* 96 h LC50 *P. promelas*	XAD SPE	Liquid–liquid acid base fractionation	HPLC	Yes	Yes
18	Municipal effluent	Microtox Photobacterium phosphoreum; 48 h LC50 *C. dubia*	C_{18} SPE	C_{18} SPE methanol–water fractionation	None	Yes	Yes
1	Municipal effluent	48 h LC50 *C. dubia;* 96 h LC50 *P. promelas*	C_{18} SPE	C_{18} SPE methanol–water fractionation	HPLC	Yes	Yes
13	Ambient water	48 h LC50 *C. dubia*	C_{18} SPE	C_{18} SPE methanol–water fractionation	None	Yes	Yes
12	Municipal effluent	48 h LC50 *C. dubia*	C_{18} SPE	C_{18} SPE methanol–water fractionation	None	Yes	Yes

GC–MS. In this way, if artifactual toxicity is inadvertently imparted to the sample or sample fraction it will be detected and the test results will be interpreted correctly.

Of equal concern is the loss of the toxicant(s) from the sample or sample fraction during the fractionation process. Because the goal of a toxicity-based fractionation scheme is isolation and concentration of an unknown toxicant, conditions that may degrade, chemically alter, or cause the toxicant to be lost from the sample are not known. Processes such as volatilization, degradation by heat, oxidation, or irreversible adsorption onto surfaces may cause the toxicant to be lost from the sample. Consequently, separation techniques and manipulations should be as benign as possible. Evaporation of the sample or sample extract to dryness, high temperatures, and other extreme conditions should be avoided if at all possible.

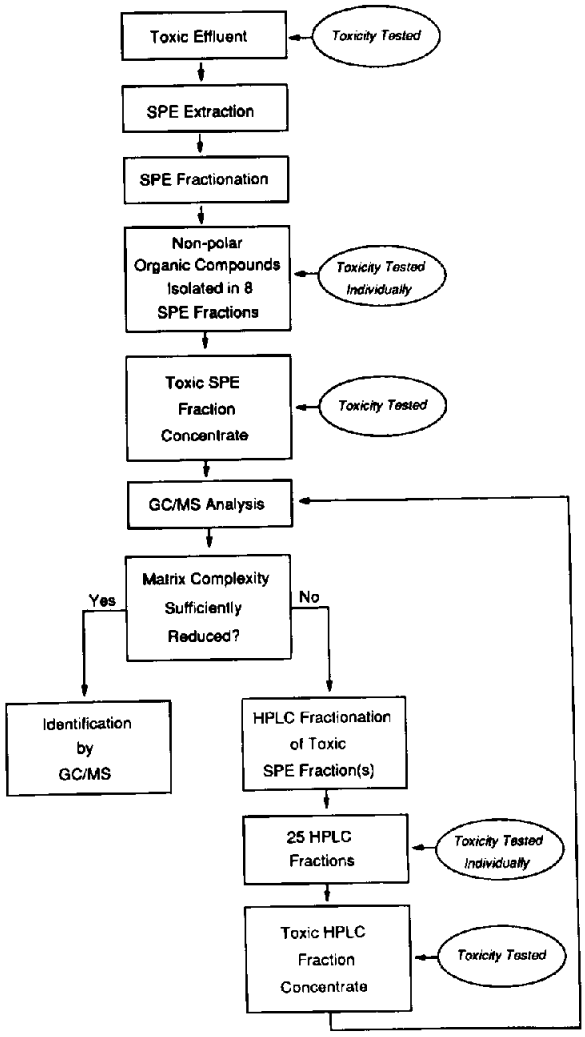

Fig. 1. Example of a toxicity-based fractionation scheme for the isolation and identification of non-polar organic toxicants.

2.1. Isolation of the non-polar toxicant from the aqueous sample matrix

Non-polar organic compounds that cause toxicity in aqueous environmental samples are often at concentration of parts per billion or less. These toxicants must first be isolated from the aqueous sample matrix and be concentrated sufficiently for identification to be possible. Two methods that are most commonly used to isolate non-polar toxicants from aqueous samples are liquid–liquid extraction using a water-immiscible solvent and extraction with a solid phase such as activated carbon, XAD resins, and octadecyl-bonded silica.

2.1.1. Liquid–liquid solvent extraction

Extraction of an aqueous sample with a water-immiscible organic solvent to isolate organic compounds has been used for many years and is a well documented method [25–27]. With this method the organic compounds are partitioned between the solvent and aqueous phases, then concentration is achieved by solvent reduction through evaporation. Diethyl ether and/or dichloromethane were used in toxicity-based fractionation of wastewaters from coal liquefaction, coal gasification, shale oil, and petroleum industries [28]. Parkhurst *et al.* [22] isolated organic compounds using solvent extraction from synthetic fuel process effluent that was toxic to aquatic biota.

Although liquid- liquid extraction has been extensively researched and there are much data available on its application to the isolation of organic compounds, there are several important disadvantages to its use in toxicity-based fractionation schemes. In general, undiluted organic solvents are toxic to aquatic organisms. Before sample extracts dissolved in these solvents can be tested for toxicity they must first be diluted with water to a solvent concentration that is non-toxic to aquatic orgnanisms. However, solvents such as dichloromethane and hexane which are used for extracting organics from water are toxic at such low concentrations, that an extract in these solvents would have to be diluted to such a large degree that any sample toxicants in the extract would also be diluted below toxic concentrations, not allowing for the toxicant to be detected. In a sense, the solvent creates such a high toxic background that the toxicant(s) cannot be detected. Therefore, to allow for toxicity testing, the solvent is commonly removed from the extract by evaporating the extract to dryness. Extracted sample components remain, it is assumed, in the residue. In addition, in a liquid–liquid extraction of an aqueous sample with a water-immiscible solvent there is some degree of mutual solubility, even if sometimes very small, between aqueous

phase and the solvent phase. Sparging of the aqueous phase with air or helium after extraction is often used to remove traces of dissolved solvent by volatilization.

It can be seen that liquid–liquid extraction has the disadvantage of imparting artifactual toxicity to both the extract and the extracted sample. Under such circumstances it is not possible to "track" the toxicity without first removing or diluting the solvent. Procedures for removing solvent toxicity from the extract and from the extracted aqueous sample run the risk of also removing volatile toxicants. In addition, the extract residue has to be redissolved in water for toxicity testing to take place and it can be difficult to be certain that all components of the residue have redissolved. The disadvantages associated with liquid–liquid extraction make it a poor choice for isolating non-polar organic compounds in a toxicity-based fractonation. Other methods, such as solid-phase extractions, avoid many of these disadvantages.

2.1.2. Solid-phase extraction

In recent years solid-phase extraction (SPE) has gained popularity as a method for removing organic compounds from water [29–31]. SPE isolates sample components by utilizing the principles of liquid chromatography (LC). Sample components are partitioned out of the aqueous sample onto the solid phase. The partition of the organic compounds onto the solid phase rather than into a water-immiscible solvent avoids many of the problems encountered with liquid–liquid extraction. Compounds adsorbed to the solid phase are recovered from the solid phase by elution with a suitable solvent. Increased concentration is achieved by eluting with the smallest possible volume that will result in good component recoveries. Wells *et al.* [30] discussed in detail the various aspects of SPE, especially with respect to toxicity-based evaluations of industrial wastewater effluents. In toxicity-based fractionation schemes, activated carbon, XAD resins (Rohm and Haas, Philadelphia, PA, USA), and C_{18} bonded silica have been used for extracting non-polar toxicant from aqueous environmental samples.

Activated carbon has a long history of use for adsorbing organic compounds from aqueous samples [32]. A disadvantage associated with its use is that recovery of adsorbed organic compounds from it is limited. This precludes isolation of the organic compounds for further fractionation and toxicant identification. For this reason, within a toxicity-based fractionation scheme, activated carbon is most often used for removing organic compounds from an aqueous solution to determine if their removal reduces toxicity. Once it is determined that removal of organic compounds also removes toxicity, then other methods such as liquid–liquid extraction have been used to actually isolate organic compounds for future fractionation. Reece and Burks [23], Doi and Grothe [15] and Gasith *et al.* [17] used activated carbon in this way.

XAD resins are macroreticular porous polymers which have been used extensively to extract organic compounds from water [31], especially for the purpose of isolating compounds for mutagenic testing [27]. XAD-2 and XAD-4 are polystyrene–divinylbenzene polymers with a high affinity for very non-polar organic compounds and XAD-7, a methylmethacrylate polymer, is efficient at extracting more polar organic compounds. Recovery of adsorbed compounds is efficiently achieved with a suitable solvent, such as diethyl ether, methanol, acetone or dichloromethane. Walsh and Garnas [24], Galassi *et al.* [16], and Jop *et al.* [14] all used XAD resins for isolating non-polar organic toxicants from effluent samples. An advantage with using XAD resins is convenience, as compared to solvent extraction, when processing large sample volumes. However, a persistent problem with their use has been resin artifacts which have been toxic to aquatic test organisms. Extensive cleaning procedures have been required to make these resins compatible with toxicity-based fractionation. Recently, however, precleaned XAD resins have become commercially available, which have not exhibited toxicity to aquatic species and are therefore an attractive alternative for use in SPE of non-polar toxicants.

Octadecyl (C_{18}) bonded to porous silica has

gained wide popularity in recent years for the SPE (C_{18} SPE) of organic compounds from water [30,33]. Recovery of the organic compounds is achieved by elution with a suitable solvent, such as those used with XAD resins. Its availability in inexpensive cartridges from several vendors has contributed to its popularity and the popularity of solid-phase extraction in general. The cartridges (columns) usually, consist of a Luer-tipped polypropylene or polyethylene reservoir packed with C_{18} bonded silica. They are available in volumes of 1 to 100 ml, packed with 100–10 000 mg of sorbent that can process from up to 100 to 10 000 ml of aqueous sample, respectively. They are convenient to use, and do not contribute artifactual toxicity to the isolation and fractionation process if simple preconditioning procedures are followed. Burkhard *et al.* [1], Mazidji *et al.* [18], Amato *et al.* [12], and Norberg-King *et al.* [13] reported the use of C_{18} SPE for isolating organic compounds from toxic effluent samples. They all reported using methanol or methanol–water solutions for recovery of the organic compounds from the C_{18} solid phase. The small volume of methanol used for these recoveries was such that sample components were concentrated 300× in the methanol eluate. In order to track toxicity, the methanol eluate requires a dilution to < 1.5% methanol–water. This dilution will render the methanol acutely non-toxic to the organisms tested in these studies, *C. Dubia*, *D. magna*, and *P. promelas*, while maintaining a concentration of 5× of the original sample. With appropriate dilution, methanol eluate from C_{18}-bonded silica can be tested for toxicity, without resorting to inherently risky procedures such as evaporation to dryness in order to remove artifactual toxicity, due to the solvent.

C_{18} SPE is a good choice for isolating organic compounds from aqueous samples in toxicity-based fractionations for several reasons: it does not contribute artifactual toxicity to the sample or sample fractions, and therefore does not interfere with toxicity tracking; non-polar organic compounds with a significantly wide log K_{ow} range can be recovered with methanol elution allowing for toxicity tracking to take place; and C_{18}-bonded silica is available in convenient to use cartridges.

2.2. Methods of fractionation

The isolation of non-polar organic toxicants from the aqueous matrix using methods, such as liquid–liquid extraction and solid-phase extraction that were discussed above, does simplify the sample matrix to some degree by separating the non-polar organic components from the inorganic and polar organic sample components. However, the non-polar extract is often a very complex mixture of non-polar organic compounds. Fractionation procedures are used to separate the mixture into subsamples (fractions) based on a chemical characteristic such as polarity. Toxicity tests are used to indicate which fraction(s) contain the toxicant(s). These toxic fractions are chemically much less complex than the original mixture making identification of the toxicant by analytical means such as GC–MS analysis possible. Fractionation methods such as acid base extraction fractionation, C_{18} SPE fractionation, and reversed-phase high-performance liquid chromatographic (HPLC) fractionation have been used for this purpose.

2.2.1. Liquid–liquid acid–base extraction fractionation

Liquid–liquid acid–base extraction fractionation is a method of liquid–liquid extraction in which the pH of the aqueous sample is adjusted appropriately to separate sample components into acid, base, and neutral fractions. This fractionation method is often incorporated directly into the initial liquid–liquid solvent extraction step described in Section 2.1.1. This procedure can be carried out on whole sample, or on an extract or residue of the sample that has been reconstituted in water. Reece and Burks [23] used this method to isolate and fractionate organic compounds from a steam volatile fraction of petroleum refinery wastewater. Walsh and Garnas [24] and Jop *et al.* [14] used it to fractionate non-polar organics isolated by XAD-4 resin from industrial and municipal effluents. Doi and Grothe [15] and Ga-

sith *et al.* [17] both proposed fractionation schemes that use acid–base extraction fractionation to isolate and fractionate non-polar organic compounds in industrial effluents, after extraction with activated carbon indicated that toxicity was due to an organic compound. Acid-base extraction fractionation presents the same problems as have been discussed with regard to solvent extraction. Fractionation of the organic compounds into solvents that are in themselves toxic at very low concentrations to aquatic organisms requires that these solvents be removed before toxicity testing can take place so toxicity can be tracked. The solvent removal techniques have the potential for chemically altering, or physically removing, the toxicants that are to be identified. Also, fractionation into three fractions is probably not adequate to sufficiently simplify the matrix to allow for the identification of an unknown toxicant by GC–MS analysis.

2.2.2. C_{18} solid-phase extraction fractionation

C_{18} SPE fractionation is an adaptation of the C_{18} SPE method for isolating organic compounds from aqueous sample. The fractionation is actually a method of recovering the organic compounds from the C_{18} SPE column into several fractions rather than into one eluate. Mount and Anderson-Carnahan [19,20] and Burkhard *et al.* [1] described in detail a fractionation method in which non-polar organic compounds are isolated and fractionated using C_{18} SPE and a methanol–water elution scheme. This is a form of reversed-phase chromatography in which organic compounds are isolated from an aqueous sample by a C_{18} SPE column, followed by sequential elution of the column with eight increasingly non-polar methanol water aliquots. This results in the non–polar organic compounds being separated into eight fractions based on their polarity. The most polar compounds elute in the fractions with the lowest methanol concentration and the most non-polar elute in fractions with the highest methanol concentrations. Burkhard *et al.* [1], Amato *et al.* [12], and Mazidji *et al.* [18] used this scheme to fractionate toxicants from municipal treatment plant effluents. Norberg-King *et al.* [13] used this method to fractionate toxicants from ambient waters. Using methanol–water solutions to generate fractions of non-polar compounds from C_{18}-bonded silica avoids many of the problems that are found with acid–base extraction fractionation. The problems inherent to immiscible organic solvents that are toxic even at very low concentrations to aquatic organisms are avoided with this method. This is a big advantage because drastic solvent-removing techniques are avoided. With this method, organic compounds with log K_{ow} values that range from about 2 to 5 are separated into eight methanol–water fractions which can be tested for toxicity after dilution to a non-toxic methanol concentration. One disadvantage of this method is that methanol while being relatively non-toxic to aquatic organisms does not efficiently elute compounds that have log K_{ow}'s much above a value of 5. Such highly hydrophobic compounds are not efficiently eluted from C_{18}-bonded silica [20] with methanol. In environmental samples such as wastewater treatment plant effluents, however, this shortcoming has not been a problem. This is due, probably, to the fact that in the process of wastewater treatment such compounds tend to be removed from solution and become associated with solids such as those found in sludge and therefore are not present in toxic concentrations in the effluents. In other kinds of environmental samples that may contain very hydrophobic compounds, such as sediment pore water, modifications to this elution method would have to be made.

2.2.3. Reversed-phase high-performance liquid chromatographic fractionation

Reversed-phase HPLC can be used to fractionate a toxic sample concentrate or SPE fraction concentrate. A reversed-phase HPLC column and an appropriate mobile phase are used to achieve separation of sample components. Column eluate is collected in discreet fractions. The isocratic or gradient elution can be designed to optimally separate the non-polar components found in a particular sample.

Jop *et al.* [14] fractionated the toxic neutral extract generated by acid–base extraction fraction-

ation. A C_{18} HPLC column and an acetonitrile–acetate buffer (pH 3.6) solvent gradient were used to effect the fractionation. Seventeen fractions were collected while the ultraviolet (UV) trace of the column elute was monitored. When the UV absorbance indicated a major peak, a fraction was collected. An interval where no major peaks emerged was collected as one fraction. An assumption is made with this method of collecting HPLC fractions that the compound causing toxicity will produce a significantly large UV absorbance peak. It may be imprudent to make that kind of assumption when fractionating an unknown toxicant because toxicity is not necessarily correlated with UV absorbance.

Burkhard *et al.* [1] used the HPLC fractionation scheme proposed by Mount and Anderson-Carnahan [20] to further fractionate a C_{18} SPE toxic fraction. This scheme uses a C_{18} HPLC column and a water–methanol solvent gradient, at a flow-rate of 1 ml/min. Fractions are collected at regular intervals of 1 min and a detector is not routinely used. This time interval for collecting fractions was chosen based on the observation that the peak width of a typical environmental sample component peak was less than 1 min. This peak width was a result of the resolving power of the HPLC column used and the flow-rate of the mobile phase. A different HPLC column and flow-rate would result in a different peak width. Because the goal was to recover a toxicant in the smallest possible volume of mobile phase, the 1-min interval was chosen. Ideally, a toxicant would elute exactly into one HPLC fraction. More probably, however, a typical sample component would elute into no more than two adjacent fractions. In practice, this has proven to be the case. Under the chromatographic conditions described, typical non-polar organic toxicants found in aqueous environmental samples, such as organophosphate pesticides, elute into one HPLC fraction or into two adjacent fractions. For this reason, when toxicity is detected in adjacent fractions it is prudent to combine them for analysis by GC–MS.

Doi and Grothe [15] proposed a toxicity-based fractionation scheme that includes fractionation by HPLC of a low-molecular-mass fraction of non-polar organic compounds, without specification of chromatographic conditions.

2.2.4. Multi-level fractionation schemes

Some aqueous environmental samples are so complex that one fractionation is insufficient to adequately isolate toxic components from the sample matrix for successful toxicant identification. In such cases a second level of fractionation can be carried out. First-level fractions that are determined to be toxic are further fractionated in the second-level fractionation. For the second-level fractination to be useful, it must be capable of further separating the components of the toxic fraction from the first fractionation. The resolving power of the second fractionation must be superior to that of the first. The fractionation scheme proposed by Mount and Anderson-Carnahan [20] and used by Burkhard *et al.* [1] provides for two or more such fractionation levels. This scheme is illustrated in Fig. 1. The first-level fractionation is carried out by C_{18} SPE, and the second and possible succeeding levels are carried out by reversed-phase HPLC. Chromatographic resolution by HPLC is far greater than by SPE. Both the SPE fractionation and the HPLC fractionation use C_{18} sorbent and water–methanol solvent elution. Toxic SPE fraction(s) are concentrated using the method of Durhan *et al.* [34]. With this method individual or combined methanol–water C_{18} SPE fractions are diluted with water to a maximum methanol concentration of 10%. This solution is then extracted with a 1-ml C_{18} SPE column. The column is then eluted with a 300-μl volume of 100% methanol. With this method fraction components are concentrated in about 200 μl of methanol. This concentrate is tested for toxicity before it is further fractionated by HPLC. In this way the presence of the toxicant(s) is confirmed before further fractionation occurs. The concentrate is injected onto the HPLC column and a solvent gradient is used to achieve the fractionation. The gradient used is dependent on which SPE fractions were toxic and are being fractionated. If toxicants eluted in the later, more non-polar SPE fractions, a gradient

could be designed that would better separate compounds that are more non-polar. Toxicity tests are carried out on the HPLC fractions. The fraction that is shown to be toxic is also concentrated by the method of Durhan *et al.* [34]. The HPLC fraction concentrate(s) is tested for toxicity before it is analyzed by GC–MS. All fractions or fraction concentrates are diluted with test dilution water to a non-toxic methanol concentration for toxicity testing. An important aspect of this scheme, as can be seen in Fig. 1, is that toxicity is tracked throughout. Every time a procedure such as fractionation or concentration is performed a test for toxicity is conducted. In this way the presence of the toxicant(s) is monitored right up to the point of GC–MS analysis. Incomplete recovery of toxicants in successive fractionation and concentration procedures can result in some loss of toxicity, but if the toxicant(s) were totally lost in the process of fractionation or concentration, GC–MS analysis of a non-toxic sample would be avoided. Conversely, since a procedural blank is subjected to the same fractionation scheme and is tested for toxicity in the same way as the sample, any artifactual toxicity will be detected.

3. GAS CHROMATOGRAPHY–MASS SPECTROMETRY

There are several techniques available for the detection of non-polar organic compounds. These include UV and infrared (IR) absorbance, nuclear magnetic resonance (NMR), and GC–MS. Toxicants isolated by HPLC fractionation are typically monitored for their UV absorbance. In our experience we have not found a relationship between UV absorbance (at any wavelength) and toxicity. In addition, there are no unique libraries available to identify unknown toxicants using their UV scans. The inherent problems with UV are the same as for IR and NMR detection; the available libraries of these type of spectra are insufficient for identifying unknown toxicants. The best technique available for the detection and identification of unknown non-polar organic compounds is GC–MS. GC provides good resolution of sample components and the mass spectra of organic compounds provide a unique form of identification. The libraries of mass spectra are quite extensive, far larger than for any other available spectra. The one disadvantage of using GC–MS is the fact that not all non-polar organic compounds are amenable to GC analysis. If the toxicant decomposes in the injection port or on the column of the gas chromatograph, the identification process is useless. In these situations an alternative inlet to the mass spectrometer, such as an HPLC system, should be used.

3.1. Preparation of toxic fractions for gas chromatography–mass spectrometry

In fractionation schemes that utilize solvent extraction or acid–base solvent extraction fractionation, non-polar organic compounds are contained in solutions of solvents such as dichloromethane that are appropriate for GC–MS analysis. Most likely, a reduction in volume is necessary to increase concentrations of non-polar components to a degree that will make detection and identification by GC–MS possible. Reduction in volume is usually carried out under reduced pressure, or under a stream of nitrogen or air.

Fractions such as those generated by SPE that are in methanol water solutions [1] or acetonitrile–acetate buffer solutions [14] cannot be injected on a gas chromatograph because the water present in the fraction can degrade fused-silica capillary columns typically used to analyze non-polar organic compounds. Mazidji *et al.* [18] dealt with this problem by combining eight methanol water fractions, two of which had been toxic to Microtox and *Ceriodaphnia*, and performing acid–base extraction to fractionate them. The acid, base, and neutral fractions in solution in dichloromethane were then analyzed by GC–MS. This strategy may be counter-productive since toxic fractions are recombined with non-toxic fractions prior to GC–MS analysis. In this way the toxicants are returned to a more complex matrix, making toxicant identification more difficult. A better strategy would be to find a way of making the toxic methanol–water fractions compatible with analysis by GC–MS and analyzing

them individually. Burkhard *et al.* [1] and Norberg-King *et al.* [13] used the back-dilution method of Durhan *et al.* [34] for concentrating C_{18} SPE or HPLC methanol–water fractions. Methanol can be injected directly into a mass spectrometer and also can be tested for toxicity with aquatic organisms upon dilution to a non-toxic methanol concentration.

Jop *et al.* [14] reextracted HPLC acetonitrile acetate buffer fractions with dichloromethane. The dichloromethane fraction extracts were brought to dryness and then reconstituted in solvents appropriate for chemical analysis, one of which was analysis by GC–MS. These fraction preparation methods used by Jop *et al.* [14] and Mazidji *et al.* [18] result in extracts that are compatible with GC–MS analysis, but incompatible with toxicity testing. As discussed earlier, solvents such as dichloromethane are not tolerated by aquatic organisms and must be removed before toxicity testing can take place. This requires procedures such as evaporation to dryness that can alter or cause the loss of sample components.

3.2. Gas chromatographic–mass spectrometric experimental conditions

Reece and Burks [23], Galassi *et al.* [16], Jop *et al.* [14], Doi and Grothe [15], Mazidji *et al.* [18], Burkhard *et al.* [1], Amato *et al.* [12], and Norberg-King *et al.* [13] described toxicity-based fractionation schemes that included analysis by GC–MS. All investigators except Doi and Grothe [15] and Mazidji *et al.* [18] described conditions under which the GC–MS data were acquired. All used fused-silica capillary columns to achieve GC separation of fraction components, and all acquired electron ionization spectra. Jop *et al.* [14] also acquired chemical ionization mass spectra. Burkhard *et al.* [1], Amato *et al.* [12], and Norberg-King *et al.* [13] used a table-top mass spectrometer, the Hewlett Packard 5790, to perform all analyses. This type of quadrupole is perfectly suitable for routine identification work. A more sophisticated mass spectrometer would be required for specialized quantitations or identifications.

3.3. Data analysis

Reece and Burks [23], Jop *et al.* [14], Burkhard *et al.* [1], Amato *et al.* [12], and Norberg-King *et al.* [13] reported performing library searches of mass spectral data using the EPA/NIH/NBS mass spectral library. Burkhard *et al.* [1] also provided a detailed strategy for interpreting mass spectral data in conjunction with toxicological data for assigning tentative and definitive identification of toxicants. This strategy is briefly outlined in Fig. 2. With Burkhard's method an internal standard is added to a small portion of the concentrate of a toxic fraction prior to injection into the mass spectrometer. This provides estimates of fraction component concentrations with internal standard quantitation. Automated library searches of the GC–MS data are carried out to provide tentative identifications of chromatographic components. Where possible the

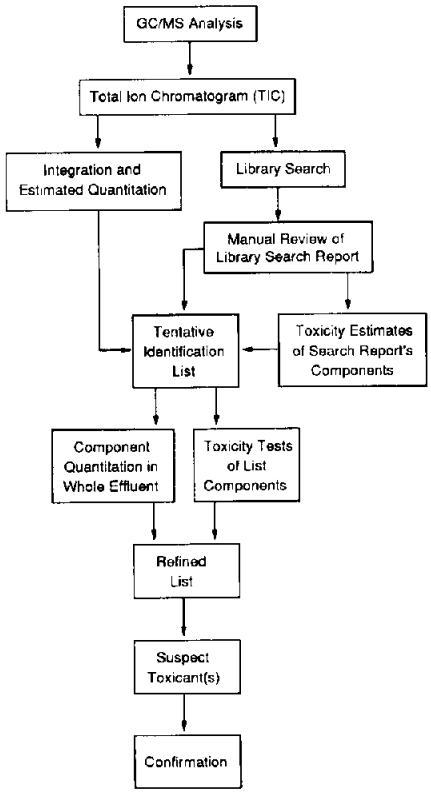

Fig. 2. Scheme for evaluating GC–MS data in conjunction with toxicological data for the purpose of assembling a list of suspect toxicants.

use of both forward and reverse searching algorithms is suggested. After automated library searching is completed, the search report along with MS data is manually examined to determine if the search report is reasonable and valid.

4. TOXICANT IDENTIFICATION

4.1. Tentative list of suspect toxicant(s)

Toxicity estimates of the tentatively identified chemicals are made by searching the literature, using quantitative structure–activity relationships or toxicity databases. Estimated concentrations and toxicity estimates are compared for each compound on the list. From this point toxicant identification becomes a process of deciding whether the suspect chemical is present in large enough quantitites to explain the observed toxicity. Due to the uncertainty of the estimations, chemicals that appear to explain the observed sample toxicity within a factor of 100 [1] are retained on the list until additional measurements have been made. Further deletions from the list are made by evaluating analytical measurements of the identified compounds in whole effluent and toxicity values that are experimentally determined. Once the list contains only suspect toxicants, then toxicant confirmation procedures should begin.

Jop *et al.* [14] took a different approach to refining the identification list. They used supplementary analytical measurements in their strategy for tentatively identifying toxicants. NMR analysis and GC with thermionic detection were carried out in addition to GC–MS analysis on the toxic extract. The results were used to eliminate compounds from the library search report and to support a definitive toxicant identification.

4.2. Definitive identification of toxicant(s)

The tentative identification list, ideally, is reduced to only a few possible suspect toxicants. For a definitive identification of the cause of toxicity in an environmental sample to be made, additional samples will have to be evaluated with the aim of confirming toxicant identity. Procedures such as correlations between suspect-toxicant concentrations and sample toxicity can be made. A strong correlation supports the toxicant identification. Spiking the suspect toxicant into the sample and measuring the change in toxicity is another approach for confirming identification; an increase in spiked sample toxicity that is proportional to an increase in suspect-toxicant concentration supports the toxicant identification. Such confirmation procedures are described in detail by Mount [21].

4.3. Alternative strategies for identification of refractory toxicant(s)

The scheme described by Burkhard *et al.* [1] will not always provide a definitive identification which can account for the observed toxicity. In such cases, additional GC–MS work can be performed under different conditions such as different temperature programs, on-column *versus* splitless injection, manual *versus* automated injection and different GC columns. It is also possible that the toxicant is at too low a concentration in the extract to be detected by the mass spectrometer. In that case it is necessary to isolate and fractionate a larger volume of sample to achieve a sufficiently large mass of the toxicant. Concentration factors as large as 100 000 are sometimes necessary to obtain high enough concentrations for identification to be possible. If lack of identification is due to an exceedingly complex sample matrix then further fractionation may be necessary. Perhaps HPLC fractionation with a different solvent gradient would isolate the toxicant in a fraction with fewer other components. It is also possible that the toxicant(s) does not pass through the gas chromatograph or that the mass spectrometer is not detecting it. In that case other modes of MS can be explored. Chemical ionization [35] rather than the more common electron-impact ionization [36] can be attempted. Other inlet options such as LC or direct probe are available [37] which use thermospray, electron-impact or chemical ionization MS techniques.

5. CONCLUSION

Toxicity-based fractionation coupled to GC–MS analysis has proved to be a useful methodology for the isolation and subsequent identification of non-polar organic toxicants from chemically complex aqueous environmental samples such as effluents and ambient waters. The goal in toxicity-based fractionation is to isolate unknown toxicants from other sample components sufficiently so that identification by analytical methods such as GC–MS becomes practical. In toxicity-based fractionation, chemical fractionation is coupled with biological detection and requires that the chemical fractionation process itself does not adversely effect the biological detector. As a result, chemical fractionation methods which introduce artifactual toxicity to the sample or sample fractions are not suitable in a toxicity-based fractionation. An example of such an unsuitable method is liquid–liquid extraction, which in its various forms is the traditional method of isolating and fractionating organic compounds from water. It requires the use of water-immiscible organic solvents that are toxic even at very low concentrations to organisms in general and aquatic test organisms in particular and thereby introduces artifactual toxicity to the sample and sample fractions. Removal of such artifactual toxicity can be very difficult if not impossible without risking the degradation or removal of the sample toxicants.

In contrast, SPE is very suitable for toxicity-based fractionation. It can extract and fractionate non-polar organic compounds without introducing artifactual toxicity to sample or sample fractions. Solid phases such as C_{18}-bonded silica and XAD resins have been used to isolate non-polar organic compounds from large volumes of aqueous sample without the use of highly toxic solvents. Recovery of a wide range of organic compounds from these solid phases can be achieved by methanol elution. Methanol is relatively non-toxic, exhibiting no acute toxicity to aquatic organisms when diluted below 1.5% in water. While C_{18}-bonded silica is the most popular of bonded reversed-phase sorbents, other non-polar bonded phases warrant consideration. There is no restriction on the solid phase that is used as long as it achieves the desired isolation and fractionation and its use does not adversely effect the biological detector, the test organism.

Fractionation by reversed-phase HPLC has much potential in toxicity-based fractionation. As with SPE, artifactual toxicity can be avoided if a water–methanol solvent system is used. Because it requires sample preconcentration to a suitable volume for injection, HPLC fractionation is better suited for second-level than for first-level fractionation. Separations by HPLC are reproducible, resolution is high, and the elution gradient can be designed to optimally separate a specific range of compounds. As a result, HPLC fractionation can achieve the high degree of toxicant isolation necessary to make toxicant identification possible. It is highly recommended as a second-level fractionation technique.

Traditionally, in SPE the solid phase has been packed into columns. An alternative form has been developed, the Empore Extraction Disk (3M, St. Paul, MN, USA) in which the bonded silica particles are enmeshed in an inert PTFE matrix, which is then formed into a disk. This new technique, claiming high flow-rates and good recoveries, shows promise for use in toxicity-based fractionations.

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REFERENCES

1 L. P. Burkhard, E. J. Durhan and M. T. Lukasewycz, *Anal. Chem.*, 63 (1991) 277.
2 A. P. Swain, J. E. Cooper and R. L. Stedman, *Cancer Res.*, 29 (1969) 279.
3 S. Schuetzle, T. E. Jensen and J. C. Ball, *Environ. Int.*, 11 (1985) 169.
4 T. J. Mast, D. P. H. Hsieh and J. N. Seiber, *Environ. Sci. Technol.*, 18 (1984) 338.
5 M. G. Nishoika, C. C. Chuang, B. A. Peterson, A. Austin and J. Lewtas, *Environ. Int.*, 11 (1985) 137.

6 B. Holmbom, R. H. Voss, R. D. Mortimer and R. Wong, *Environ. Sci. Technol.*, 18 (1984) 333.

7 M. R. Samoiloff, J. Bell, D. A. Birkholz, G. R. B. Webster, E. G. Arnott, R. Pulak and A. Madrid, *Environ. Sci. Technol.*, 17 (1983) 329.

8 W. R. West, P. A. Smith, G. M. Booth and M. L. Lee, *Environ. Sci. Technol.*, 22 (1988) 224.

9 D. F. Wiemer, *Rev. Latinoam. Quim.*, 16 (1985) 98.

10 M. A. Quilliam and J. L. C. Wright, *Anal. Chem.*, 61 (1989) 1053A.

11 L. P. Burkhard and G. T. Ankley, *Environ. Sci. Technol.*, 23 (1989) 1438.

12 J. R. Amato, D. I. Mount, E. J. Durhan, M. T. Lukasewycz, G. T. Ankley and E. D. Robert, *Environ. Toxicol. Chem.*, 11 (1992) 209.

13 T. J. Norberg-King, E. J. Durhan and G. T. Ankley, *Environ. Toxicol. Chem.*, 10 (1991) 891.

14 K. M. Jop, T. Z. Kendall, A. M. Askew and R. B. Foster, *Environ. Toxicol. Chem.*, 10 (1991) 981.

15 J. Doi and D. R. Grothe, in G. W. Suter II (Editor), *Aquatic Toxicology and Environmental*, Vol. 11, ASTM STP 1007, ASTM, Philadelphia, PA, 1989, p. 123.

16 S. Galassi, C. Battaglia and L. Vigano, *Chemosphere*, 17 (1988) 783.

17 A. Gasith, K. M. Jop, K. L. Dickson, T. F. Parkerton and S. A. Kaczmarek, in W. J. Adanis, G. A. Chapman and W. G. Landis (Editors), *Aquatic Toxicology and Hazard Assessment*, Vol. 10, ASTM, Philadelphia, PA, 1988, p. 204.

18 C. N. Mazidji, B. Koopman, G. Bitton, G. Voiland and C. Logue, *Toxic. Assess.*, 5 (1990) 265.

19 D. I. Mount and L. Anderson-Carnahan, *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures*, EPA/600/3-88-034, U.S. Environmental Protection Agency, Duluth, MN, 1988.

20 D. I. Mount and L. Anderson-Carnahan, *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures*, EPA/600/3-88-035, U.S. Environmental Protection Agency, Duluth, MN, 1989.

21 D. I. Mount, *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures*, EPA/600/3-88-036, U.S. Environmental Protection Agency, Duluth, MN, 1989.

22 B. R. Parkhurst, C. W. Gehrs and I. B. Rubin, in L. L. Marking and R. A. Kimerle (Editors), *Value of Chemical Fractionation for Identifying the Toxic Components of Complex Aqueous Effluents, Aquatic Toxicology*, ASTM STP 667, ASTM, Philadelphia, PA, 1979, p. 122.

23 C. H. Reece and S. L. Burks, in R. D. Cardwell, R. Purdy and R. C. Bahner (Editors), *Isolation and Chemical Characterization of Petroleum Refinery Wastewater Fractions Acutely Lethal to Daphnia Magna, Aquatic Toxicology and Hazard Assessment: Seventh Symposium*, ASTM STP 854, ASTM, Philadelphia, PA, 1985, p. 319.

24 G. E. Walsh and R. L. Garnas, *Environ. Sci. Technol.*, 17 (1983) 180.

25 T. L. Voke and I. H. Suffet , in C. E. Van Hall (Editor), *Measurements of Organic Pollutants in Water and Wastewater*, ASTM STP 686, ASTM, Philadelphia, PA, 1979, p. 47.

26 J. Eichelberger, E. H. Kerns, P. Olynyk and W. L. Budde, *Anal. Chem.*, 55 (1983) 1471.

27 I. H. Suffet and M. Malaiyandi (Editors), *Organic Pollutants in Water, 188th Meeting of the American Chemical Society, Philadelphia, PA, Aug. 29–31*, 1984 (*Advances in Chemistry Series*, Vol. 214), American Chemical Society, Washington, DC, 1987, p. 201.

28 H. L. Bergman, R. A. Kimerle and A. W. Maki (Editors), *Environmental Hazard Assessment of Effluents, Proceedings of a Pellston Environmental Workshop, Valley Ranch, Cody, WY, Aug. 22–27, 1982*, Pergamon Press, New York, 1986, Ch. 9, p. 92.

29 E. Chladek and R. S. Marano, *J. Chromatogr. Sci.*, 22 (1984) 313.

30 M. J. M. Wells, A. J. Rossano, Jr. and E. C. Roberts, *Anal. Chim. Acta*, 236 (1990) 131.

31 S. A. Daignault, D. K. Noot, D. T. Williams and P. M. Huch, *Water Res.*, 22 (1988) 803.

32 R. L. Jolley, *Environ. Sci. Technol.*, 15 (1981) 874.

33 G. A. Junk and J. J. Richard, *Anal. Chem.*, 60 (1988) 451.

34 E. J. Durhan, M. T. Lukasewycz and J. R. Amato, *Environ. Toxicol. Chem.*, 9 (1989) 463.

35 A. G. Harrison, *Chemical Ionization Mass Spectrometry*, CRC Press, Boca Rotan, FL, 1983.

36 F. W. Karasek, O. Hutzinger and S. Safe (Editors), *Mass Spectrometry in Thermospray Environmental Science*, Plenum Press, New York, 1985.

37 R. A. Hites, *EI Handbook of Mass Spectra of Environmental Contaminants*, CRC Press, Boca Raton, FL, 1985.